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Interpretation of Real-Time PCR Results for Hepatitis C Virus RNA When Viral Load Is Below Quantification Limits▽

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Hepatitis C virus RNA quantification results obtained in 18 laboratories using real-time PCR methods with 10 negative samples and 22 sample dilutions (viral loads of 0.5 to 500 IU/ml) showed a score of correct results of up to 93.5%. However, 55.6% of the laboratories did not follow the recommendations for the interpretation of their results, leading to ambiguous conclusions.

The determination of hepatitis C virus (HCV) RNA levels has become an essential part of patient care, from early diagnosis of infection to treatment monitoring. Thus, the interpretation of HCV RNA quantification results should be done in accordance with precise and standardized diagnostic criteria. The use of molecular assays for HCV RNA quantification based on real-time PCR has become more widespread since they are able to detect levels of viral nucleic acids below 100 IU/ml (1–4). Moreover, these methods show high analytical sensitivity, making it possible to detect viral RNA below the lower limit of quantification (LoQ). Since the goal of antiviral treatment is full eradication of HCV from the blood (8) and since a diagnosis of acute infection may sometimes be based on the detection of very low viral loads (VLs) (5, 6), it is essential to distinguish the true absence of circulating viral RNA from a low and unquantifiable VL.

The Action Coordonnée 11 group of the Agence Nationale de Recherches pour le SIDA initiated, with 18 laboratories, a study focused on the interpretation of the results of real-time PCR assays showing VLs below the threshold of the assay. The objective of this study was to point out the differences in the interpretation of such samples in order to allow virologists to give unambiguous results for low HCV RNA VLs to physicians.

A panel including 32 coded samples was sent to 18 participating laboratories. This panel was composed of three groups of samples. Group 1 included 10 HCV RNA-negative samples collected from blood donors who tested negative for hepatitis B surface antigen, HCV and human immunodeficiency virus antibodies (Prism; Abbott, Rungis, France) and for HCV RNA (COBAS *Taq*Man HCV; Roche, Meylan, France). The other two groups were made up of dilutions of two specimens. Specimen 1 was genotype 1a, with an HCV RNA VL of 150,000 IU/ml (COBAS *Taq*Man 48), and specimen 2 was genotype 2a with a VL of 5,200 IU/ml (COBAS *Taq*Man 48). Group 2 included 12 samples corresponding to three dilutions of specimens 1 and 2 with theoretical VLs of 0.5, 5, and 10 IU/ml present in duplicate. Group 3 included eight samples corresponding to three duplicated dilutions of the same specimens with VLs of 50 and 100 IU/ml, one dilution at 500 IU/ml for specimen 1 and one at 300 IU/ml for specimen 2.

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TABLE 1. Results obtained with CAP/CTM and ART for the 32 samples in the panel

Test	No. of labs	No. of samples/total (%)				Score (%), range[e]
		Group 1[a]	Group 2[b]	Group 3[c]	Total[d]	
CAP/CTM	6	59/59 (100)[f]	72/72 (100)	55/60 (91.7)	186/191	97.4, 93.7–100
ART	12	117/118 (99.1)[g]	135/144 (93.7)	120/120 (100)	372/382	97.4, 93.5–100
Both[e]	18	176/177 (99.4)	207/216 (95.8)	175/180 (97.2)	558/573	97.4, 93.5–100

[a] Negative, 10 samples.
[b] VLs, 0.5 to 10 IU/ml, 12 samples.
[c] VLs, >50 IU/ml, 10 samples.
[d] Thirty-two samples.
[e] The score is the percentage of correct results among the expected results. The expected results were negative for group 1, negative or positive but under the LoQ for group 2, and positive with a VL in the range of quantification for group 3. The range of scores according to laboratory is shown in parentheses.
[f] One lab provided nine results.
[g] Two labs provided nine results.

Of the 18 participating laboratories, 6 used COBAS Ampliprep/COBAS *Taq*Man HCV 2 (CAP/CTM) from Roche Diagnostics (range of quantification, 43 IU/ml to 6.9×10^7 IU/ml; limit of detection [LOD], 15 IU/ml) and 12 used Abbott M2000sp/Abbott Realtime HCV PCR (ART) from Abbott Diagnostics (range of quantification, 12 IU/ml to 10^8 IU/ml). According to the two manufacturers, the following three categories of results were possible: (i) RNA not detected when HCV RNA is under the detection limit of the test, (ii) HCV RNA detected when the value is below the lower quantification limit, and (iii) HCV RNA detected when the value falls within the linear quantification range of the assay. The samples were assayed in single testing according to the manufacturer's instructions.

The results were analyzed at two levels. The first one aimed to analyze the results obtained after the PCR process. In this case, the expected results were "negative" for samples in group 1, "negative or target detected but not quantifiable" for samples in group 2 (to take into account Poisson distribution, which can give alternatively positive or negative results when viral RNA approaches the detection limit of the technique), and "target detected with a quantifiable VL" for samples in group 3. For each laboratory and each group of samples, the score was calculated as the percentage of correct results among the number of expected results. The second level of analysis, which only focused on samples in groups 1 and 2, aimed to evaluate the interpretation of participants for results from a "clinical" point of view. These clinical interpretations were classified into two categories. The first included the conclusions recommended by the manufacturer ("absence of detectable HCV RNA" when the result of the PCR process was negative and "presence of RNA" when the result was positive); the second included all other ambiguous interpretations suggesting the presence of HCV RNA in negative samples (for example, "under the LoQ") or its absence in positive samples (for example, "negative, under the LoQ").

For samples in groups 1, 2, and 3, 59, 72, and 60 results were provided with CAP/CTM and 118, 144, and 120 were provided with ART, respectively. The global score of correct results was 97.4% (range, 93.7 to 100%) for CAP/CTM and 97.4% (range, 93.5 to 100%) for ART (Table 1). Two of the 6 laboratories using CAP/CTM and 8 of the 12 laboratories using ART gave 100% correct results. The highest score was obtained for CAP/CTM in groups 1 and 2, as 100% of the samples included in these groups were correctly classified, while ART had a better score in group 3, with 100% of the results correct. CAP/CTM failed to correctly quantify five samples included in group 3, giving positive results but with a VL under the LoQ. ART provided one false-positive result in group 1 and overestimated nine samples in group 2. In group 2, the results obtained with CAP/CTM in a total of 72 cases were "not detected" in 23 cases (32%), "RNA detected but below the LoQ" in 49 cases (68%), and "quantifiable RNA" in none; those obtained with ART in a total of 144 cases were "RNA not detected" in 50 cases (35%), "RNA detected but below the LoQ" in 85 cases (59%), and "quantifiable RNA" in 9 cases (6%).

The two investigated HCV real-time PCR assays had good specificity, since only one false-positive result (with a sample with a VL under the LoQ) was observed among the 177 tests performed with HCV-negative samples.

Table 2 shows the mean VLs obtained with the two assays in samples in group 3. Coefficients of variation ranged from 23.2% to 37.6% for ART and from 19.4% to 44.6% for CAP/CTM. Despite the underestimation of the VL in five samples in group 3, CAP/CTM gave significantly higher VLs than ART in samples with VLs above 100 IU/ml ($P < 10^{-3}$). Furthermore, among the samples containing a VL below the LoQ, ART provided a slightly higher proportion of negative results than CAP/CTM (35% versus 32%).

Regarding the interpretation of the results analyzed for samples in groups 1 and 2, only 8 (44.4%) laboratories (4 using CAP/CTM and 4 using ART) followed the manufacturer's recommendations for the interpretation of the results obtained and 10 (63.6%) gave ambiguous interpretations for both groups ($n = 6$) or alternatively for negative ($n = 1$) or positive results ($n = 3$).

Despite the indisputable performance of the two PCR assays, even though some slight differences have been observed between the two methods, a heterogeneity was noted in the interpretations given by the participating laboratories, irrespective of the method used, especially with critical samples (negative or containing a VL close to the LoQ). Indeed, 55.6% of the labs did not follow the manufacturers' recommendations for the clinical interpretation of their results. However, the study was performed out of any clinical context, and this certainly contributed to the ambiguity of the conclusions provided by some labs, especially for "negative" samples. In any case, these ambiguous conclusions underline the necessity of the knowledge of the clinical context to give a reliable interpretation of such results.

TABLE 2. Mean VLs of samples with more than 50 IU/ml (group 3)

Specimen and theoretical VL (IU/ml)	CAP/CTM			ART			P^a
	No. of determinations	Measured VL (IU/ml)	CV (%)	No. of determinations	Measured VL (IU/ml)	CV (%)	
1							
50	11	40	30.6	24	40	37.6	NS[b]
100	12	99	19.4	24	74	24.1	$<10^{-3}$
500	6	807	20.7	12	333	29.5	$<10^{-4}$
2							
50	10	34	44.6	24	40	36.1	NS
100	10	71	41.1	24	84	23.2	NS
300	6	396	32.8	12	242	29.2	$<10^{-3}$

[a] P = probability obtained after analysis of variance (F test) comparing the mean VLs obtained with the two assays. The difference was considered significant when P was <0.05.
[b] NS, not significant.

Distinguishing the absence of HCV RNA and its presence at an unquantifiable RNA level remains a delicate point largely depending on the clinical context. When an early infection is suspected, especially when anti-HCV antibodies are not yet detectable, detection of viremia, even at a low level, is crucial in order to initiate early antiviral therapy. Furthermore, "eradication" of HCV, which is the goal to be achieved to assess treatment efficacy (7), remains to be defined. It is thus important for clinicians to have reliable biological results reflecting the reality of the eradication status. In this matter, the true question is whether we need to reach a "zero" viremia level or an undetectable RNA level that would be arbitrarily determined as the detection limit of the assay to consider that HCV eradication has been attained. This issue should be resolved with the availability of more-sensitive PCR methods, but today it remains of importance.

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